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Thesis

Bacterial Variation with Special Reference to the Pneumococcus

by

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(B.S., College of the City of New York, 1944)

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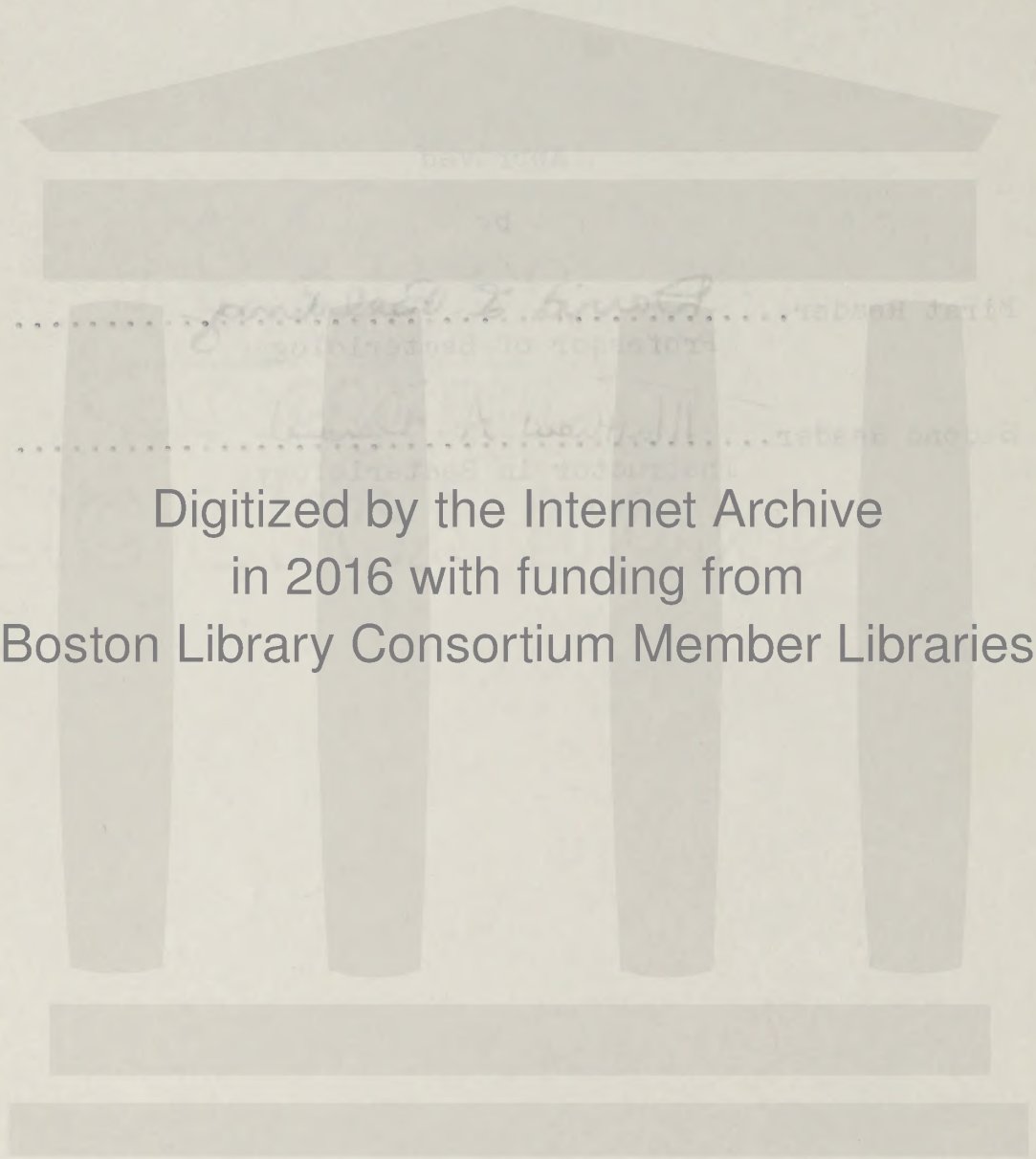

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INTRODUCTION

During the latter half of the nineteenth century, which saw the rise of scientific bacteriology and immunity, the subjects under investigation in these sciences were unicellular and multicellular microscopic organisms. In common with all embryonic sciences, the major portion of these early investigations was directed towards the classification of those organisms in either the plant or animal kingdom. The first accepted view was that they belonged in the latter, and as such they were referred to as "animalculi," little animals. However, further investigations of such phenomena as cell walls, plasmolysis, and various morphological characteristics led eventually to their present classification, in the plant kingdom.

Ferdinand Cohn asserted, "They form the boundary line of life; beyond them, life does not exist, so far at least as our microscopic expedients reach; and these are not small." Indeed, the microscopic size of bacteria led the physicist to consider them as simple colloidal systems, while the chemist thought of them as "bags of enzymes." This assumed primitiveness was not confirmed biochemically until the end of the century when Winodgradsky(1887) announced that certain microorganisms were capable of synthesizing their own protoplasm from mineral salts and carbon dioxide. Here was a phenomenon, the synthesis of highly complex organic substances from simple inorganic salts, which might well be interpreted, in view of their occurrence in living

organisms, as the beginning of life on earth.(Dubos)

Further cytological studies during the next fifty years have revealed a complexity of structure in these "primitive" organisms which is comparable to that found in the higher plant and animal forms. They have further brought about the possibility of an anatomical comparison between these bacterial cells and those which together make up the highly complex structures of higher plants and animals. The processes which take place within the cells of both types, assuming that we may draw a line of phylogenetic differentiation between them, have been found to be similar in a great many respects. Although we are at present incapable of determining the exact nature of many of these metabolic phenomena occurring within various living cells, we are, nevertheless, by means of carefully controlled experimental studies of various substances which we may identify within the cell, and as belonging to a particular cell, able to draw certain conclusions concerning these metabolic processes, by analogy with certain similar phenomena which have occurred naturally in vitro or in vivo, or which have been made to occur in vitro or in vivo by purely artificial and synthetic, or very nearly artificial and synthetic means. Furthermore, it has been shown (McCarty, Avery, Feulgen, and others) that bacterial cells contain within themselves certain chemical substances which are known to be fundamental, not only to bacterial genera but to cells of higher plants and animals, without which these cells cannot carry on their life cycles. They have been shown to require certain essential substances for growth, these substances

being identical with the vitamins required for normal animal and plant growth. They have been shown to require certain amino acids as a minimum essential for survival. Further, and by far the most important analogy which has been drawn so far, is the observation that many of the properties of bacterial cells are identifiable with similar properties, not only of viruses but also of the chromosomes and genes of plant and animal cells.

As we consider histology and microscopic anatomy to be the study of hundreds of different types of cells, with many individual characteristics and functions, analogously we may consider bacteriology to be the study of many different types of cells. But whereas it is almost impossible, except under very carefully controlled experimental conditions, to isolate any single animal or plant cell from its usual environment and cause it to continue to grow and exhibit its normal characteristics outside of the animal organism, on the other hand it is possible to study one particular bacterial cell, in reference to its growth, means of fission, and its various biochemical, physical, morphological and other properties. It is moreover possible to observe in a short time the effect of an environmental change in an evolutionary phase of growth on a particular type of cell, due to its rapid growth and frequent generation. Thus it may be possible to study the effect of varied oxygen supply upon a culture of a bacterium, e.g., Staphylococcus aureus, which has been grown for a long time in a controlled oxygen environment. It is then possible to observe the properties of subsequent generations of this bacterium, and so study the results of environmental adapt-

ivity, not over a period of a thousand years but in a few days.

Further, as it has been found that certain bacterial and animal cells have substances in common, it is possible to infer the metabolism of the animal cells, and also the role of these apparently fundamental components of the cell, by means of in vitro experiments with the bacterial cells. One of the common properties of bacterial and animal cells is that of producing a mutation-like phenomenon at the rate of 1 per 10^5 or 10^6 cell divisions. In the animal, when this variant occurs, it generally finds conditions unfavorable for its growth and continued existence, therefore it passes out of the anatomical picture. In the case of the variant bacterial cell, it is possible to isolate such cells when they occur, and to examine their differential properties as compared with the parent strain, and with other bacteria. Although it is not possible, except with gamma rays, etc., to increase the incidence of the occurrence of these mutations, it is nevertheless possible to increase the rate of survival of these mutations by growing the parent strain on a medium which will favor the growth of the variant, while at the same time supplying the parent strain with sufficient environmental factors necessary for its growth. Depending upon the factors that have caused the variation, and upon the type of variation, there may or may not be a reversion to the parent strain.

It is the purpose of this paper to present one aspect of the possibilities of applying the methods and investigations of bacterial variation and transmutation. Transmutation enters into the analysis to a greater extent than variation, the former

term implying a variation which is transmissible from one generation to the next, in series. Our intent is to apply these methods of investigation to the problems of the mechanisms of metabolic processes within the animal cell. This side of the problem has been approached from a purely bacteriological and immunological point of view by many investigators, Alloway, Avery, Dawson, Dochez, Dubos, Griffith, Heidelberger, McCarty, Riemann, and others, through the study of experiments connected with pneumococcal variations, both permanent and temporary.

Before proceeding to this topic, it is necessary to point out the various aspects of bacterial variability, as seen in mutations exhibited by species other than Diplococcus pneumoniae. This will be the purpose of the first section of this paper, namely, an analysis of bacterial variants, both naturally occuring and artificially induced. By this means we shall be able to set down a groundwork of definitions and illustrations from which we can better proceed to the problems of pneumococcal transformation.

BACTERIAL VARIABILITY

Bacteriology is a statistical science. It is not the study of individual organisms; all the facts which together make up that body of knowledge which we call bacteriology are derived from the study of a great many generations of a bacterial type, represented by many millions of organisms. By subjecting this large number of similar organisms to the identical experimental conditions, which is the limit of our experimental methods at present, we are able to observe the properties of a large number of the organisms present. Thus our statistical data are drawn from negative as well as positive results. For example, when we say that the organism Escherichia coli has the property of fermenting lactose, we base this conclusion upon the visual evidence that when a colony of this organism has been isolated and transplanted to a tube of phenol red broth containing lactose, we observe a color change from red to yellow, indicating that acid has been formed in the tube; we also observe gas in the collection tube. Statistically we may say that Escherichia coli is capable of fermenting lactose. However we know that in the fermentation tube there are many millions of organisms present, some of which are obviously capable of fermenting the carbohydrate present. But we cannot say that every organism present has that property. No one has yet devised a bacteriological technique whereby the fermentative properties of a single cell of the species Escherichia coli, or of any other bacterial species can be tested.

On this basis we might also say that the majority of the cells of Escherichia coli are incapable of fermenting lactose, but that a sufficient number of naturally occurring variants possessing that property are produced in the normal growth curve of the organism during the 24-hour incubation period to make the phenomenon visible. On the other hand, we have the organism Escherichia coli mutabile, which will not ferment lactose. In this case the statistical evidence is on the negative side. We can assume that there are not present sufficiently great numbers of naturally occurring variants which are capable of fermenting lactose to give visual evidence of that fact.

It appears that all bacterial cells are capable of producing mutant forms during their normal growth cycles. These variants may or may not be permanent.(Deskowitz) They may transmit the properties which bear witness to their variance from the parent strain, or they may revert to the parent strain, or they may give rise to both the parent strain of the variant and to the variant itself. These naturally occurring variants cannot be artificially controlled, but they seem, according to Dubos, to occur regularly within the life cycle, and can therefore be predicted with surprising accuracy.

The mutabile strain of Escherichia coli which was mentioned before will usually revert after a time to the lactose-fermenting form. Similarly the communis strain of the same organism, which does not ferment sucrose, gives rise to the communior strain, which does ferment this sugar, and by a study of these two strains and their alterations, the mutation from one to the other

may be adequately predicted.

Aside from these naturally occurring mutations, we are mainly concerned with those variants which are artificially produced and which are generally stable and permanent. From a study of these mutations, effected by physiological, biochemical, structural, serological and antigenic methods of variation, we have been able to obtain a groundwork of knowledge which can be applied in the field of medical bacteriology to epidemiological studies of various diseases, and to the cytological and histological studies of higher plant and animal cells and of their metabolic processes and their structure.

PLEOMORPHISM AND MONOMORPHISM

In the early days of bacteriology one of the first manifestations of bacterial variability to be observed and studied was that of pleomorphism and monomorphism. It was thought that there was only a handful of bacterial types, and that these exhibited marked variation in their morphology, and in their biochemical properties. This early claim was eventually displaced, and its rise to prominence was attributed to faulty techniques, particularly in the manner of obtaining and maintaining pure cultures of bacteria. It was then seen that the bacteria presented a great multiplicity of distinct types.

The view which is generally accepted by present-day bacteriologists is that the bacteria represent a phase of degeneration of the higher organisms, i.e., they have through evolution and environmental adaptivity lost certain properties which alone serve to distinguish them from the higher plants, particularly the blue-green algae and the true or green algae, and so on up the phylogenetic tree. It is possible in a general way to classify the bacteria not only according to the properties which in the main each genus or species may exhibit, but also to classify them by the properties which they may lack, and which serve to differentiate them from each other, and also from the higher plant organisms.

We find that the majority of the bacteria will retain their form from generation to generation, depending upon the culture medium which is used, and aside from slight variations which may

be compared in man to the fact that some are tall or short, and some are fat and thin, though their environment has not varied sufficiently to warrant an explanation of these deviations by reason of a degeneration or an evolution of the species. However we do find certain bacteria which when grown on a suitable substrate will, upon microscopical examination, exhibit varying forms. This may be shown by growing a single-cell culture, the technique of which is very well defined(Avery, R.C. and Leland, S.J.), to eliminate the possibility of contamination. One example of this form of variation, which would seem to come under the heading of naturally occurring mutants, is Pasteurella tularensis. This organism is commonly found in the smear in both coccoid and bacillary forms. In young cultures both forms may be found, the coccoid form predominating. The coccoid form is generally found alone in older cultures. This would appear to indicate that the bacillary form is the variant.(Zinnser) The mechanism of this mutation may possibly be explained in the following manner. It is generally known that bacillary forms tend to elongate due to an internal axially disposed force which tends to counteract the rounding effect of surface tension on the cells.(Dubos) On this basis we may assume that the surface tension of the culture medium generally used for the isolation and cultivation of Pasteurella tularensis, a cystine-glucose broth, will be sufficiently low to cause the naturally occurring mutant forms to survive at a rate sufficient to be detected by a microscopic smear. We must then further assume that upon aging the surface tension of the medium is sufficiently increased, due

to the accumulation of secreted products from the organisms so that only the coccoid forms are found. However, this hypothesis cannot in any way be validified upon observation of cultures of this organism grown upon agar slants made from the same essential ingredients, since these cells also exhibit coccoid and bacillary forms.

We can only say in this particular case that the mutation is a natural phase of the cytomorphosis of the cell during its normal growth curve. This would appear to be the more valid explanation.

It appears that many bacteria exhibit slight, but occasionally marked variations in shape during their normal growth curve. These variations consist of differences in the size and contour of the bacterial cell, the loss of certain structures, such as capsules and flagella, variations in normal grouping, and variations in the internal structure of the cell. Some of these naturally occurring variations are illustrated below, along with other variations which are interdependent upon each other. This interdependence of variations will be discussed later on, indeed throughout the paper, for it appears from all the data in the literature that a single variation within a species is almost unknown.

The streptococci generally show a wide variation in size at practically all points along their normal growth curve, the diameter ranging from 0.4 to 1.0 micron; these differences may be observed within a single chain, large and small cocci being adjacent to one another, showing that the one was the result of

the fission of the other. Thus we can differentiate this fact from a possible observation of two distinct chains of cocci, in each of which the cocci are of equal size, while the diameter varies from one chain to the other.

The Pneumococcus Type III may be found in the normal diplococcal arrangement of the genus, but almost invariably exhibits chain formation, which makes it difficult to distinguish the organism from the streptococcus. This chain formation in turn is generally associated with a variation in the shape of the organism. Instead of the normally occurring lanceolated form, pneumococcus Type III tends to be ovoid in shape, resembling not only the streptococcus, but also the genus Neisseria, from which, fortunately, we can as a rule differentiate it by means of the gram stain. Photomicrographs taken of pneumococcus Type III colonies on blood agar plates show this chain formation at the periphery of the colony; the pneumococci are seen to be almost spherical in shape.(Bisset)

The genus Corynebacterium is the most striking example of pleomorphism. The forms vary from the evenly staining slender bacilli, through barred and granular varieties of clubbed and globular forms. The occurrence of these forms is quite easily predicted during the normal growth curve of the organism.(Morton) There has been, moreover, no correlation between any of the various morphological forms and the virulence of the diphtheric bacillus, although the granular type seems to predominate in clinical diphtheria.(Belding and Marston; Morton)

The flagellated bacilli are most often observed without the

flagella. This is the general case, because these structures, being very delicate, are often lost due to handling during the preparation of a stained smear. This, of course, is not a true variation. However, flagellated bacilli, e.g., Salmonella paratyphi, may be caused to show a non-flagellated variant by growing the organism in serum containing antibodies directed against the flagella. This gives rise to a non-flagellated, non-motile and non-specific strain of Salmonella paratyphi.(Arkwright and Pitt) This method of producing artificial variations in flagellated or encapsulated species has been used to a great extent in the study of bacterial dissociation, and will be mentioned again later, particularly in reference to the transformation of pneumococcal types. This variation associates itself with others which are interdependent upon it. Thus, in the experiments described by Arkwright and Pitt, the flagellated form of Salmonella paratyphi was determined by its agglutination in homologous immune serum containing antibodies directed against the "H", or flagellar antigens. Those organisms so agglutinated were observed to form smooth, dome-shaped colonies, show uniform turbidity inbroth culture, and absence of agglutination in 0.85% salt solution. This was designated as the smooth, or "S" form, according to Griffith. Those organisms showing irregularity of the surface and margins of the colonies, granular growth in broth culture, and agglutinability in 0.85% salt solution were designated as rough, or "R" forms. By the experimental method described above Arkwright and Pitt cultivated and described variant forms of Salmonella paratyphi and Eberthella typhosa. The smooth

forms of these organisms were agglutinated in immune sera containing the homologous "H" antibodies, while the rough forms were not agglutinated by these sera, but did react when placed in immune sera containing the antibodies directed against the homologous "O", or somatic antigens. It was possible to imply from these observations that the smooth forms of the organisms were flagellated, while the rough forms had lost their flagella. Similar experiments were done with Klebsiella pneumoniae(Julianelle) and various pneumococcus types.(Griffith; Riemann) But whereas it has now been found possible to cause the reversion of R pneumococci to the original type-specific S forms by growing the R form in anti-R serum, and by other methods which will be described later, it has not been possible in vitro in the case of the flagellated organisms. The explanation of this phenomenon is probably the fact that the R to S dissociation of the pneumococcus is due to the elaboration of a polysaccharidal capsule, which appears to be brought about easily by certain transforming and inducing substances present in normal and immune sera. On the other hand, to convert the R form of flagellated organisms to the S form it is necessary that a flagellum be elaborated by the organism, a change which would appear to involve a fundamental change in the cell structure of the bacterium. This does not appear feasible at the present time.

It has been noted(Julianelle and others) that S forms of flagellated organisms agglutinate spontaneously when treated with homologous immune sera, whereas the corresponding R forms agglutinate quite slowly, giving in higher concentrations a

fluffy precipitate, and in the higher dilutions a granular precipitate, which is difficult to read without a lens.

It very rarely happens that a bacterial cell will exhibit a single phase of variation. Variations involve changes in the structure of the bacterial cell. These changes may be visible or invisible, and further may be permanent or reversible, either by natural or artificial experimental methods of cultivation. The change may be merely the loss of a capsule, or it may be a change of reactivity towards the gram stain. No matter what it may be, it usually affects the entire definitiveness of the properties of the cell. In 1922, De Kruif described an experiment involving the mutation of the bacillus of rabbit septicemia from the highly virulent D form to the almost avirulent G form. He observed, aside from the difference in virulence exhibited by the two mutant forms, differences in colonial formation and appearance, and differences in the acid agglutination optima of the two varieties. He further observed that this last phenomenon implied a distinct change in the bacterial protoplasm of the G form, which would seem to be the most fundamental mutation so far described. He showed that growing a pure-line strain of the D organism in diluted rabbit serum enhanced the tendency of mutation to the G form. On the other hand, all attempts to grow a pure-line strain of the G mutant in undiluted rabbit serum, which inhibits the tendency of the D form to change to the G variety, and thus bring about a reversion to the D form failed, further confirming the concept of a fundamental change in the bacterial protoplasm.

COLONIAL VARIATION

Colonial variation appears to be the most fundamental manifestation of the variability of bacteria in regard to seemingly permanent changes, because of the fact that it is generally associated with mutations of almost every variety, one being excepted, the artificial transformation of the pneumococcus from one type to a virulent pneumococcus of another specific type, which is distinct and transmissible. We shall mention this later. Colonial variation within a species indicates the occurrence of mutant forms. Motile varieties may often be distinguished from non-motile strains, the colonies of motile organisms usually showing spreading growth, or colonies with irregular margins, as compared with the usually distinct and small colonies of the non-motile varieties, e.g., Proteus vulgaris.

Non-motile bacteria which tend to multiply in chain formation may exhibit non-chain forming mutants. The presence of chains within a colony may give a veined or coiled appearance. This has been shown in photomicrographs(X300) by Bisset. Colonies which show such formation are encountered in certain species of streptococcus; the viridans strain gives a swirled vortical appearance; Bacillus anthracis shows the "medusa-head" colony formation characteristic of that species.(Bisset)

Colonial formation is largely dependent upon the structure of the organisms of which the colony is composed, and to a lesser extent upon their biochemical and physiological properties. Colonies of encapsulated organisms, as the pneumococci and Klebsi-

ella pneumoniae, are moist and glistening, of stringy consistency, and possessing even surfaces and margins. It has been pointed out that the encapsulated S pneumococci should be classified as forming M, or mucoid colonies.(Dawson) This type of colony is typified by the encapsulated form of Klebsiella pneumoniae, which upon nutrient solid media forms rather large, flat, moist, glistening colonies with even margins. The organisms in these colonies are rather short forms, even the bacillary organisms. The consistency of the colony depends upon the amount and the nature of the capsular substance secreted by the organism. Klebsiella pneumoniae elaborates a large amount of capsular substance, which is easily recognized under the microscope by ordinary staining methods, and its colonies are quite viscous. On the other hand, the pneumococcus elaborates less capsular material, hence the mucoid phase is less viscous. It should be noted here, and will be referred to extensively later, that the type-specific pneumococci which are designated as the S form of the species are actually the M, or mucoid variant, according to their colonial morphology. Colonies of virulent, encapsulated pneumococci are small mucoid varieties. Also, the R form of avirulent non-capsulated pneumococci form typical smooth colonies of the S variety. It is possible to obtain typical R colonies of pneumococci(Dawson; Shinn) with a wrinkled appearance, showing filamentous growth. This is found to be the ultimate R cultural phase of the pneumococcus.

It has been found possible to relate colonial organization to the method of growth of the organisms.(Bisset) It is noted

that in smooth colonies of motile organisms the colonies are made up of long individual organisms. This is attributed to the sliding movement of the organisms past each other directly after fission. Colonial morphology has been correlated with two other types of post-fissional movement, snapping and whipping, and the sliding movement mentioned above. Organisms occurring in the "medusa-head" colony have been shown to exhibit a snapping post-fissional movement. This was first associated with the rough dissociation phase of Bacillus anthracis.(Bissett) It was also shown to occur in the rough colonies of Escherichia coli. Rough colonies seem to show a characteristic arrangement of the bacteria when examined microscopically, by reason of the snapping post-fissional movement; they are generally found lying side by side in small bundles. The smooth variant forms of coliform organisms show no characteristic arrangement within the colony. Following fission these organisms separate and slide partially past each other. The corynebacteria show a whipping post-fissional motion which gives rise to vortical colonies exhibiting growth in chains similar to colonies of streptococci. It has been noted(Gause) that Bacillus mycoides contains an identifiable protoplasmic property which causes the threads to grow clockwise or counter-clockwise, giving rise to dextral and sinistral mutations.

It has been observed that practically all bacterial cultures exhibit several types of morphologically distinct colonial forms, including carefully prepared single-cell cultures. These become stabilized through several generations and settle down in one

phase or another.

Colonial morphology may usually be associated with other structural and biochemical properties of bacteria. These organisms whose virulence depends upon their possession of a specific capsular substance, such as Klebsiella pneumoniae and the type-specific S pneumococci, form M, or mucoid colonies. Hence mucoid colonies may be judged as being composed of encapsulated and virulent organisms. Similarly, when these two species are grown in immune sera directed against their respective specific soluble substances(Heidelberger and Avery), non-capsulated, avirulent bacterial cells arise which form typical smooth colonies.

In the case of organisms whose virulence is associated with their flagella, as in the cases of Salmonella paratyphi and Eberthella typhosa, the virulent flagellated motile variants are shown to form typical smooth colonies(there are instances of rough variants). The avirulent, non-flagellated, non-motile mutants exhibit rough colonial formation(smooth colonies have been noted). Smooth colonies in general indicate the virulent, type- or group-specific variation of the species; rough colonies are almost invariably avirulent and species-specific.(Arkwright; Bruner and Edwards; Morgan and Beckwith)

The diphtheria organism shows a variation in its colonial organization which appears to be correlated with varying degrees of virulence.(Morton) Three strains of the virulent form of Corynebacterium diphtheriae are recognized by colonial formation on blood-tellurite medium. These are designated, according to the original theory of their varying degrees of virulence, mitis,

intermedius, and gravis. However, it has been found that these original designations do not truly connote the relative virulence of the three strains. The morphology of the gravis strain consists of uniformly staining short forms, which have been found in some clinical cases, especially in the more severe ones. Its exact role in clinical diphtheria is still not yet clear. The gravis strain shows two main antigenic groups, each of which has been found as an epidemic strain over wide areas. The morphology of the mitis strain shows long slender forms with meta chromatic granules. This type is the predominant one in clinical diphtheria in this country. It exhibits a great deal of antigenic diversity, a fact which may account for its greater overall virulence and occurrence.(Belding and Marston; McLeod) The intermedius type exhibits the barred clubbed variety of the bacillus, and it is an antigenically homogeneous group. The three strains also exhibit some variation in their biochemical properties, in that the gravis strain will ferment starch and glycogen, while the mitis and intermedius will not.

The species Klebsiella pneumoniae was observed (Julianelle) to be divided into three distinct serological types, A, B, and C. These three types, in their virulent, encapsulated phase, are agglutinated only in their homologous antisera. The virulent encapsulated variants of each type form M, or mucoid colonies on nutrient agar plates. When these colonies are transplanted for several weeks, a translucent mutant arises in each case. The number of transplants necessary to bring about the degradation varies with each type. Type A needs about four weekly transplants

while Type B needs six to eight weeks, and Type C will usually show the mutant form after only two weekly transplants. If the procedure of subculturing is continued, the smooth, translucent variants give rise to a still further dissociated phase, the rough variant. The translucent colony variant is smooth, and composed of short non-capsulated rods, resembling those of the parent strain in each type. The translucent variant has always been the first to appear during the process of natural dissociation. The rough variant is invariably derived from the translucent type and produces a rough appearing colony which is composed of long non-capsulated rods and filamentous forms.

The M, or mucoid phase of Type A is designated as As, the smooth phase as At, and the rough or R form as Ar, those of Type B as Bs, Bt, and Br, and of Type C, Cs, Ct, and Cr. Agglutination tests were performed using antisera prepared against each of the nine mutants of Klebsiella pneumoniae. It was shown that all the mucoid forms were agglutinated only by their respective homologous immune sera. In each type the smooth variants were agglutinated by their respective immune sera, and each showed cross-agglutination with the immune serum prepared against the mucoid phase of the homologous type. The rough variants Ar and Br were agglutinated only by anti-Ar and anti-Br sera, respectively. The Cr mutant was agglutinated by its homologous immune serum, and showed cross-agglutination with anti-Ct serum. Julianelle (1937) has reported that the rough variants of Klebsiella pneumoniae of different serological types cross-react. The results of investigations by Randall (1939) do not confirm the occurrence

of cross-reactions among the rough variants of encapsulated Klebsiella pneumoniae. It is probable that the use of two different methods of inducing dissociation explains the different results, and as shown by Julianelle even rough variants derived from the same serological type of Klebsiella pneumoniae are not always serologically identical.

Hence we may now describe three distinct types of bacterial colonies, each of which appears to be linked with one or more variations of the structural, and possibly of the biochemical properties of the organism.

The M, or mucoid colony consists of encapsulated, generally virulent organisms, giving specific agglutination in homologous immune serum, and no cross-agglutination with immune sera of any other type within the species. The colony is viscous, moist, glistening, and possesses even margins; without animal passage it has a tendency to dissociate towards a more "stable" form. In this class are found the S forms of the pneumococcus.

The S, or smooth colony consists of non-capsulated, generally avirulent organisms. This type is found only in those species which exhibit a mucoid phase, which is true of most pathogenic bacteria. The other group of organisms forming S colonies are generally flagellated organisms, the Shigella and the Coccaceae being the principal exceptions. These forms, like the mucoid variants, tend to dissociate to more "stable" forms. The S phase forms smooth, generally moist colonies, with even surfaces and margins. The R form of the pneumococcus falls under

this classification of colonial variation.

The R, or rough colony is made up of non-capsulated, or non-flagellated, avirulent organisms (except Bacillus anthracis, which is virulent in the rough colony phase). The organisms in this phase are species-specific only (exceptions are noted in the Enterobacteriaceae). The ultimate R cultural phase of the pneumococcus is the true R form of the species.

It is difficult to draw up such general definitions for each of these dissociative phases, for there are within a species or a species type, and from one species to another, variations which lie outside these general rules. There are the encapsulated S and R variants of Klebsiella pneumoniae (Julianelle), the smooth non-motile and rough motile variants of the Colon-Salmonella organisms (Arkwright; Zinsser; and others), and the virulent R forms of the pneumococcus.(Griffith) These, along with all the other naturally occurring and artificially induced, and transformed mutations, go to make up a very heterogeneous group of organisms indeed. There are so many factors of environment, both purposed and accidental, that the subject must more or less be studied not only from the standpoint of individual species alone, but of single variations within species, their causes and their effects, and the factors which may be introduced into any system to cause reversions or further dissociations, or perhaps still more fundamental bacterial changes.

VARIATIONS OF THE PNEUMOCOCCUS

The study of the pneumococcus in its many phases of dissociation has made up a large part of the literature on the phenomena and mechanisms of bacterial variation. The experiments set forth by the various workers (Alloway; Avery; Brown; Dawson; Dochez; Dubos; Eaton; Goebel; Griffith; Heidelberger; McCarty; Mudd; Paul; Riemann; Shaw; Shinn; Sia; Stryker) represent studies of both the naturally occurring and the artificially induced mutants. The variations to be discussed are those of colonial formation, immunochemical specificity, and the transformation of pneumococcal types; also, some of the methods which have been used in the course of these investigations and which are applicable to other biological problems will be discussed.

The work on the pneumococci as a group, subsequent to the discovery and cultural isolation of the organism, by Pasteur in 1881, and by Fraenkel in 1886, respectively, was begun in 1905 by Collins, Hiss, and Park and Williams, who did extensive studies of the biochemical properties of the organism, and examined the behavior of the various strains with which they worked toward immune sera. The work received its greatest impetus from the observations of Neufeld and Levinthal who in 1909 separated all of the then-known strains of pneumococci into four distinct serological groups, Types I, II, and III, and the heterogeneous Group IV, by means of the capsular swelling(quellung) reaction. They observed that when pneumococci were mixed with immune sera prepared by injecting rabbits with living inocula of

various strains of pneumococci, the capsules of the organisms became swollen in certain sera, those which had been prepared against the homologous organism. They were able to show that those organisms which showed capsular swelling in one immune serum showed it only in that serum, i.e., the reaction was type-specific. This method was disregarded for a number of years due to the inability of subsequent workers to duplicate the original results. Recently (1943), Mudd, Heinmetz, and Anderson have been able to repeat the original experiments, using immune sera prepared from rabbits, and have prepared a series of electron photomicrographs of the reaction in an attempt to explain more fully its mechanism. At the present time this method is widely used, due to improved methods of preparing the specific immune sera from the blood of rabbits, and it has replaced the formerly used methods of Krumwiede and Sabin. The original Types I, II, and III are still so designated, while the heterogeneous Group IV has been separated into over seventy types, by means of absorption techniques. All the types so examined and classified were gram positive cocci, occurring in pairs or in chains, and exhibiting identical structural properties, i.e., the possession of a capsule, virulence to mice, solubility in bile salts and similar chemical compounds, inulin fermentation, and the production of methemoglobin from oxyhemoglobin.

In his studies on patients suffering from pneumonia, Griffith (1928) described two colonial variants of pneumococci isolated from both the sputum specimens of his patients and the pleural exudates from experimental mice which he was using for

typing. The one was the virulent, encapsulated organism which formed moist, glistening mucoid colonies. These he designated as the S form of the pneumococcus, adopting Arkwright's terminology. He also described a rough variant which was avirulent and non-capsulated. These pneumococci formed smooth, moist colonies on blood agar plates, and were designated by Griffith as the R form of the pneumococcus. This nomenclature has led to a great deal of misconception on the part of workers who are not familiar with the organisms involved.

It has been demonstrated (Avery; Dawson; Griffith; Reimann; and others) that the S form of colony is invariably composed of virulent type-specific organisms, and that this property, i.e., type-specificity, is transmissible indefinitely through mouse passages and subcultures on normal serum media. There is a tendency on the part of these S organisms to dissociate towards the intermediate SR and Rs forms, and towards the ultimate R cultural phase (Shinn) when grown on non-vital media. However, several animal passages of any organisms showing this tendency will usually suffice to restore their virulence and type-specificity, and the formation of typical mucoid colonies. In contrast, the R pneumococci show species-specificity only, i.e., they are agglutinated by the same sera regardless of the S type from which they were derived, and transmit this property in series through innumerable transplants. However, if such forms are passed through mice, there may be a reversion to the S form from which they were derived. This will depend upon the degree of dissociation. It was shown (Alloway; Dawson and Sia) that R

pneumococci which were grown in media containing serum against the species antigen of the pneumococcus showed a tendency to revert to the original virulent, encapsulated S form from which they were derived.

All S pneumococci are virulent when injected into mice, and the organisms which are isolated from the pleural exudates upon autopsy (Heidelberger) are shown to be serologically and colonially identical with thoses of the original inoculum. Conversely all R pneumococci are non-pathogenic to mice. An exception to this rule was noted by Griffith, who reported finding only R pneumococci in the pleural exudates of a mouse which had died after having received an injection of living R pneumococci together with a heat-killed vaccine of Type III S pneumococci. No explanation has been given.

All type-specific, virulent and encapsulated S pneumococci which form typical mucoid colonies on blood agar plates, and will retain the aforementioned properties through an indefinite number of mouse passages. However, when these organisms are subjected to a lengthy series of transplants and subcultures in broth media and on blood agar plates, they tend to dissociate towards the R form, thus losing their virulence for mice, their type-specificity, and their ability to elaborate the capsular substance. Depending upon the degree of dissociation, these properties may be recovered by various means, e.g., growth in anti-R serum. The R form, on the other hand, when grown on normal serum-containing media, retains the R phase, and show no tendency to revert to the S form. There may however be a further

dissociation towards the ultimate R cultural phase.

In 1933 Dawson described a rough variant colony of the pneumococcus which exhibited marked pleomorphism. Many elongated coccoid and coccobacillary forms were seen. Organisms from early cultures were entirely gram positive, but smears taken from older transplants showed a marked variation in reactivity towards the gram stain. He noted that in a long series of transplants these pleomorphic cells had a tendency to revert to the rough variant of the R form and also ultimately to the parent strain of the S form.

The rough variant of Dawson is colonially and morphologically identical with the ultimate R cultural phase of the pneumococcus, which was described by Shinn in 1937. However, Shinn reported that there was no tendency towards reversion of this ultimate R form, i.e., highly dissociated, to typical R or S pneumococci. He also reported that this highly dissociated variant was no longer soluble in bile. These differences would seem to indicate that the ultimate R cultural phase of Shinn was further removed both morphologically and physiologically, and in regard to colonial formation from the virulent, encapsulated mucoid form of the pneumococcus than the variant described by Dawson. It appears that the ultimate R cultural phase is the true rough variant of the species, since it has reached a high state of stability, and cannot be reverted by any means to either the variant R form or to the mucoid type-specific S form; furthermore it seems to have undergone a fundamental change in its protoplasmic and cell-wall structures, being no longer sub-

ject to autolysis (Eaton; Shinn), and showing marked variability in reactivity to the gram stain.(Dawson) These last facts, and its loss of the power to elaborate a capsular substance, as well as the power to put into operation an enzyme or other physiological system capable of producing such a substance, even when a transforming material is added to the system, point to a fundamental change in the biochemical and physiological processes within the pneumococcal cell.

Dawson's further experiments on the colonial variations of the pneumococcus led to his study of the relationships among the colonial variations of Streptococcus hemolyticus, an organism related to the pneumococcus. In this instance, as in the former, it was found that three types of colonies could be isolated from a lengthy series of transplants, beginning with virulent organisms forming typical mucoid colonies on blood agar plates. These were quickly dissociated to the smooth, non-capsulated form, since hemolytic streptococci have been shown to elaborate a capsular substance only for a very few hours after seeding and streaking a known virulent strain on artificial media. Further transplants degraded the smooth phase still further and the R phase was obtained. These three colonial types corresponded relatively in virulence, encapsulation, and the colonial morphology to the three types of colonies of the pneumococcal mutations. These variations were further correlated to the colonial mutants of a wide range of bacterial species, Salmonella paratyphi (Arkwright and Pitt), Klebsiella pneumoniae (Julianelle), Bacillus anthracis (Bisset), Streptococcus viridans (Bisset), etc.,

In summation of the colonial variants of Diplococcus pneumoniae, the M, or mucoid phase of virulent, encapsulated, type-specific, gram positive lanceolated diplococci, which form moist glistening, stringy colonies with even surfaces and margins. The S, or smooth phase consists of usually avirulent, non-capsulated, group-specific gram positive diplococci which form smooth, moist colonies with even surfaces and margins, generally a little smaller than the colonies of the mucoid phase. These pneumococci retain the property to revert to the mucoid phase under the proper conditions, or the R form under further conditions conducive to such further dissociation.

The R, or rough phase of the pneumococcus consists of avirulent, non-capsulated, species-specific organisms which show great variation in their reactivity to the gram stain, sometimes gram positive, sometimes gram negative, and exhibit marked pleomorphism, ranging from the typical lanceolated cocci occurring in pairs to long filamentous forms. These pneumococci form rough, dry colonies on blood agar plates, with rough surfaces and irregular margins. The R form of the pneumococcus is bile insoluble, and has lost the property of reverting, or being induced to revert to the S or M phases by any means.

TRANSFORMATION OF PNEUMOCOCCAL TYPES

In 1909 Neufeld and Levinthal were able to divide the species Diplococcus pneumoniae into three distinct types and one large heterogeneous group, on the basis of serological procedures. They designated these subdivisions as Types I, II, and III, and Group IV. Types I, II, and III reacted only with their homologous antisera, though Types I and III did show cross-agglutination in dilutions up to 1 to 400. Group IV was a large heterogeneous mixture of types which at that time these workers were unable to classify on any serological basis.

In 1915 Avery studied a number of pneumococcal strains which showed agglutination in Type II anti-pneumococcal serum. He showed that these strains, which he subdivided into three subgroups and classified under Type II, were serologically distinct from Type II pneumococcus. Type II anti-pneumococcal serum after having been absorbed with Type II pneumococci was unable to agglutinate any of the three subgroups. When the same serum was absorbed with any of the pneumococci of the three subgroups, it was still capable of agglutinating either of the other two subgroups, and also Type II pneumococci. It appears here that these strains, which Avery was able to classify in three distinct serological groups, were part of the heterogeneous Group IV. These had previously remained unclassified because of their cross-agglutinative reactions with Types I, II, and III antisera. The agglutination, absorption and protection techniques used by Avery have now been applied to a great number

of pneumococcal strains which were lumped together under Group IV; this group has been shown to contain over seventy serologically distinct types.

In 1916 further mutations of Type II pneumococcus were noted by Stryker. Mutants were brought about by growth in homologous immune serum, and variations in virulence, bile solubility, inulin fermentation, capsule elaboration, and antigenic properties were observed. It is worthy of note that of all the variations that have been observed in association with the pneumococcus from time to time, the only one which has been seen by direct observation of the organism has been the presence or absence of the capsule. The importance of the capsular substance will be demonstrated shortly.

In 1917 Dochez and Avery demonstrated in the cell-free filtrates of pneumococcal cultures a specific soluble substance which reacted with anti-pneumococcal sera. They showed that this substance of itself was non-antigenic. It was further shown that this substance differed with each S type of pneumococcus, thus correlating it to the serological specificity of the several types. It was shown that each of these specific soluble substances could be isolated from the cell-free filtrates of pneumococcal cultures, and also from the bacteria themselves after they had been freed of all culture medium material, followed by pneumococcal autolysis by various means.(Heidelberger and Avery; Goebel; Brown)

It is now generally believed that the specific soluble substance of the pneumococcus which is found in the cell-free fil-

trates of cultures, in urine, serum, and ascitic fluids of infected individuals is identical with the capsular substance of each type of pneumococcus. On this basis Heidelberger (1923, 1924, 1925, 1927, 1936) isolated the specific soluble substances of pneumococcus Types I, II, and III from cell-free filtrates. He showed these three substances to be similar in chemical composition, all of them being essentially polysaccharides of the order of starch and glycogen, though giving the reactions of neither of these two compounds when treated with iodine reagent. They were shown to yield glucose on hydrolysis, along with other carbohydrate compounds, among which were an aldobionic acid, in the case of Type III, and an acetylated amino sugar, in the case of Type I. It appeared that each of these compounds, including glucose, which probably enters in as a factor due to its type of linkage with the other substances, were at least a part of the determinative factors which gave to each pneumococcal type its particular nature and antigenic properties. These substances, as obtained, were subjected to a variety of qualitative and quantitative chemical and physical tests. They were shown to differ from one another in content of carbon, hydrogen, oxygen, and nitrogen, and in the optical activities of their aqueous solutions. They appear to be protein-free substances, giving none of the usual qualitative tests for proteins and amino acids. Their serological specificity has been shown by means of the precipitin reaction with immune sera. At the present time some seventy-five or more distinct serological types of the pneumococcus have been demonstrated by various techniques. Each of

these specific soluble substances has been isolated and subjected to chemical and physical analyses.(Blake; Brown)

The capsular polysaccharide of the pneumococcus has been shown to be responsible for many of the properties attributed to the organism; thus variations in the capsule cause correlated variations in other properties. While it has been demonstrated that the virulence of the pneumococcus is not attributable to either an exotoxin or endotoxin, it is readily shown that the organism is extremely invasive. This high degree of invasiveness is largely due to a high resistance to phagocytosis within the animal body. The resistance of the organism to phagocytosis is attributed to the presence of the capsule, which is a highly polymerized and very viscous polysaccharide. It is difficult to say, though it is generally assumed to be so, that the polysaccharide elaborated by the organism is a true capsule, since the substance is also found in cell-free filtrates, being water-soluble. It may be that this substance merely collects around the organism following its elaboration and secretion through the cell wall and clings to the wall by reason of its own high viscosity and its internal cohesion. Encapsulated pneumococci when freed of any culture media may then be washed free of the capsular polysaccharide, similar to Klebsiella pneumoniae, and in contrast to Mycobacterium tuberculosis, the waxy capsule of which appears to be an integral part of that organism, and can only be removed by such drastic procedures as treatment with boiling alcohol. On the other hand, serological evidence points to the fact that the capsular polysaccharide alone is incapable

of stimulating the production of antibodies, when it is freed of the rest of the intact pneumococcus. It has also been shown that the injection of lysed cells which still hold the protein substance of the pneumococcus in combination with the polysaccharide are also incapable of stimulating antibody production. The cell protein alone will not evoke antibodies against type-specific pneumococci, or against the cell protein combined with the polysaccharide; further, the cell protein will not react with any type-specific anti-pneumococcal serum. On the other hand, the polysaccharide alone, or in combination with the cell protein will give a precipitin reaction with antisera produced by the intact cells.

Thus we have seen that the capsular polysaccharide of the pneumococcus is responsible for the resistance of the organism to phagocytosis within the animal body, for the virulence of the organism by way of enabling it further to invade the host, and for the specificity of the seventy-five serological types. The other variations, bile solubility and inulin fermentation, while they appear to be associated with variations in capsular elaboration, do not seem to be directly dependent upon it, as there seems to be a change within the bacterial protoplasm which may account for such mutations.

The last variation associated with the inhibition of capsular elaboration to be discussed is that of colonial formation of the pneumococcus. Although this subject has been taken up previously in some detail, it will be necessary to repeat certain salient facts pertinent to a further explanation of these muta-

tions. The pneumococcus dissociates into three distinct phases, mucoid, smooth, and rough forms. The colonial distinction of these three forms may be made macroscopically by observation of the texture and shape of the colonies. When observed microscopically the organisms appear encapsulated in the mucoid phase, and rarely in the smooth phase; the non-capsulated organisms are found in the smooth and rough phases. The smooth and rough phases may be differentiated from each other microscopically by showing that the smooth phase consists of regular morphological forms, i.e., diplococci, while the ultimate rough phase consists of long rods and filamentous forms, which often appear gram negative. The further serological distinction was made that the smooth phase could be reverted to the mucoid phase, while the rough phase no longer retained this property.

We have also seen previously that when the smooth phase, or so-called R form of the pneumococcus was reverted to the mucoid phase, it again required the serological specificity of the S type from which it originally dissociated.(Dawson; Stryker) However, it has been found possible, by means of the intermediate smooth phase, or R form, to convert pneumococci of one type to those of another type, and this specificity may then be transmitted in series, or may again be reverted to the original type, or to still another specific type, again by way of the intermediate smooth phase.

In 1923 Griffith described the effect of culturing type-specific pneumococci in homologous immune sera. From these cultures he obtained the R forms of the respective specific types.

These rough phases were identical with respect to their biological properties and serological manifestations, except for the fact that upon reversion to their original mucoid phases they regained only the type-specificity of the parent strain from which they had been derived. The work on the transmutation of pneumococcal types has stemmed mainly from the observations of Griffith (1928) and Riemann (1929). In an epidemiological study undertaken in 1928 on a number of patients suffering from pneumonia, Griffith studied the pneumococcal types in sputum specimens collected from these patients. Among them he found pneumococci of Types I, II, and III, of Group IV, and a species-specific type which has been described previously, and which was designated by that investigator as the R form of the pneumococci. After this preliminary sputum typing, Griffith proceeded to pass the organisms through mice, and then to type the organisms obtained in the peritoneal washings of the animals at autopsy. He found that when certain sputa which had been identified as containing Type II organisms were injected into mice, the animals appeared to have died from an infection of Type III S pneumococci, which were obtained from the peritoneal washings in almost pure culture.

This apparent change in type was also associated with a definite variation in the virulence of the organisms. This was additional evidence, showing that the Type II S pneumococci had acquired not only the serological specificity of Type III S organisms, but had acquired the virulence of Type III. The change in virulence is undoubtedly due to two properties of the pneumococcus, first, that the Type III pneumococcus is a more

virulent organism than Type II, and second, upon which the first may very well depend, that Type III pneumococcus elaborates more capsular polysaccharide than Type II, and in its virulent phase it tends to forms chains of encapsulated diplococci, thus combining the invasive powers of both the streptococcus and the pneumococcus with an increased resistance to phagocytosis.

Riemann had found previously (1925) that type-specific S pneumococci grown in vitro in immune serum of the homologous S type gave rise to a mutant form consisting of non-type-specific non-capsulated R forms of the pneumococcus, which retained those properties upon subsequent transplantation and subculture in either the homologous S type immune serum or in normal serum. He further noted that when these non-specific R pneumococci were injected into a mouse together with a heat-killed suspension of S pneumococci of the homologous type from which the R mutant had been derived, the mouse died of an infection with living type-specific S pneumococci. Upon serological examination these S pneumococci were identified as being of the same type as the organisms in the heat-killed suspension. The organisms in the shspension were shown to be non-viable, and the living R pneumococci were demonstrated to be of themselves avirulent to mice, by suitable animal and culture medium controls. It has been shown that the virulence of the pneumococcus is to a large extent dependent upon the individual capsular substance. Conversely, the R pneumococci have been shown to be non-capsulated and avirulent. The former property appeared to be dependent upon the latter. It has also been noted that the R pneumococci show no

agglutination or swelling reactions in any of the type-specific sera. It was subsequently shown that all R pneumococci, regardless of the S strain from which they were derived, are agglutinated only in anti-R serum, regardless also of the derivation of the R strain used to prepare that serum.

From the above data the assumption was made that there must be in the heat-killed suspension of S pneumococci a substance which is capable of inducing the elaboration of capsular polysaccharide by living R pneumococci, which of themselves did not possess this property. It was further assumed that this inducing substance was type-specific, i.e., it induced the elaboration of the capsular polysaccharide of the specific type from which the transforming substance was derived, and of that type only. This was shown in further experiments (Avery; Dawson; Dawson and Sia; Heidelberger; McCarty; McLeod and McCarty) in which R pneumococci derived from a number of different S types, I, II, III, IV, and VIII, were reverted to their original type-specificity, and furthermore, could be converted to any of the other types, by the use of cell-free filtrates of the desired type, and by the use of the intermediate smooth phase. The procedure used was that originally propounded by Griffith. Dawson injected living R pneumococci derived from Type II pneumococci into mice, together with large amounts of a heat-killed vaccine of Type III S pneumococci. When these organisms were isolated from the pleural exudates (Heidelberger found this to be a more convenient and more abundant source of the organisms than the peritoneal washings used previously) subsequent to the death of

the animals, they were shown to agglutinate in Type III antiserum, and to give pure cultures of Type III S pneumococci when cultured in serum media. Similarly, when a heat-killed vaccine of Type I S pneumococci was substituted for the Type III S suspension in the above experiment, the organisms isolated were shown to be Type I S pneumococci. In neither case were any S pneumococci of Type II isolated from the animal exudates.

It is therefore seen that S pneumococci of Type II have actually been transformed, by means of a specific transforming substance, into S pneumococci of Type I, and of Type III. In each case the transformation was brought about through the use of the intermediate smooth phase of the organism. A number of workers (Alloway; Dawson; Dawson and Sia), by the use of cell-free filtered extracts of Type III S pneumococci, succeeded in effecting the transformation of Type II S pneumococci into Type III S pneumococci *in vivo* and *in vitro*; the *in vitro* transformation was brought about through the use of the intermediate R form, as were the transformations *in vivo*. There is non substantiated data showing the direct conversion of S pneumococci of one type to S pneumococci of another specific type. Barnes and Wight have described the spontaneous transformation of Type V S pneumococci to Type II S pneumococci *in vivo*, but this work has never been corroborated.

By the injection of a small inoculum of living R forms of Type II pneumococcus, together with large amounts of a filtered cell-free extract of Type III S pneumococci into a mouse, it has been possible to obtain a pure culture of Type III S pneumococci.

Similarly, Type II S pneumococci were converted to the R phase by growing the organisms in Type II anti-pneumococcal serum. The organisms thus obtained were then transplanted to a culture medium containing a filtered cell-free extract of TYPE III S pneumococci. Similar transplants were made of the Type II R organisms to media containing filtered cell-free extracts of other type-specific S pneumococci. In each case the Type II R pneumococci were converted to the S form of the homologous serological type of the filtered extract used in the culture medium.

These experiments led various investigators (Avery; Heidelberger; MacLeod; McCarty) to the conclusion that there must be present in the filtered cell-free extracts of S pneumococci a substance, which has been produced by the specific type of organism present in such an extract, and which is capable of inducing the elaboration of the homologous type-specific capsular polysaccharide by R pneumococci, regardless of the type derivation of the latter. It may further be concluded that R pneumococci, while they do not possess any of the several transforming substances <u>in situ</u>, nevertheless retain the property of utilizing such a substance, when it may be added to the system. This may at some future time be shown to be due to the activation of an enzyme, or of an enzyme system, which is responsible for the elaboration of the capsular polysaccharide.

There is, however, the aforementioned ultimate R cultural phase of the pneumococcus, described by Shinn, and by Dawson. It was found in many instances that when S pneumococci were transformed, or "degraded", to the R form, and were subsequently

injected into mice, together with heat-killed vaccines of either homologous or heterologous S pneumococci, there appeared to be no reversion or transformation of R to S pneumococci. An increase in the amount of extract or vaccine used for injection seemed to have no effect upon the reversion or transformation. Shinn continued his experiments by growing the organisms in homologous anti-S serum, thus transforming S to R pneumococci. He continued this degradation through a long series of transplants in this anti-S serum. He finally reached a point of dissociation at which he had obtained a pure culture of avirulent and non-capsulated R pneumococci, which could not be transformed to any other type-specific S pneumococci, nor could it be reverted to its original type-specificity by any means. No capsule formation could be noted, or induced by any means, nor was there any virulence for laboratory animals. This was termed the ultimate R cultural phase of the pneumococcus, and as such was discussed in detail in the section dealing with the colonial variations of the pneumococcus.

NUCLEOPROTEINS AND BACTERIAL GENETICS

It was not until 1944 that a more extensive study was made by Avery, McLeod, and McCarty of the transforming substance that was known to be present in pneumococcal cultural extracts. The work of the above investigators was directed mainly towards the isolation, purification, and examination of the chemical and physical properties of the transforming material, as well as its physiological behavior. They started with crude extracts of cultures of Pneumococcus Type III. (This work has since been extended to two other pneumococcal types, namely, Types II and VI.) These crude extracts from heat-killed pneumococci are complex mixtures of components of the bacterial cells themselves, along with the substances originally present in the culture medium. It was found that the removal of proteins, lipids, the somatic and capsular polysaccharides, and the ribonucleic acid fraction from extracts of Type III cultures had no effect upon the transforming activity of the residual extract material. Accordingly, they proceeded to remove these components as quantitatively as possible, the proteins by the chloroform method, the somatic carbohydrate by fractional alcohol precipitation, the capsular polysaccharide by digestion with a specific enzyme which hydrolyzes it, and the ribonucleic acid by digestion with the enzyme ribonuclease, or by alcohol fractionation.(Levene and Bass)

The product obtained after these preliminary extractions had been carried out possesses practically all of the biological

activity of the original crude extract. It was readily soluble in aqueous and saline solutions, giving clear, colorless solutions which were highly viscous even at relatively low concentrations, and which showed high bifringence. The material is precipitated by alcohol in the form of a mass of fibrous threads, which loses none of its transforming activity upon repeated alcohol precipitation. Qualitative tests for both proteins and ribonucleic acid are negative. On the other hand, the diphenylamine reaction for desoxyribonucleic acid is strongly positive. The quantitative elementary tests carried out on several different samples of the active material show the content of carbon, hydrogen, oxygen, nitrogen, and phosphorus to be comparable to that found in pure samples of sodium desoxyribonucleate. The nitrogen-phosphorus ratio of 1.67 conforms closely to that of the sodium salt, and further eliminates the possibility of protein contamination. Absorption spectra curves in the ultraviolet region showed a maximum high absorption at 2600 A, which is known to be characteristic of nucleic acids.(Caspersson; Mirsky) Solutions of the material lose none of their biological activity after treatment with the enzyme ribonuclease. These data lead therefore to the conclusion that the transforming activity of the material is associated with a nucleic acid of the desoxyribose type.

Solutions of the purified substance were not observed to give precipitin reactions with Type III anti-pneumococcal serum of high titer, in the dilutions characteristic of the serologically active substances, though slight reactions were noted in

very low dilutions.

Analysis of samples of the purified material in the Tiselius apparatus and in the Svedberg ultracentrifuge both give evidence pointing to the presence of a homogeneous substance of high molecular weight. The ultracentrifuge gave a sedimentation constant corresponding to a molecular wieght of approximately 1,000,000, which is characteristic of nucleic acids of the desoxyribose type. The Tiselius electrophoretic data also showed a single sharp boundary, with which the transforming activity appeared to be associated.

The transforming activity of the material was tested quantitatively with serial dilutions of R pneumococci derived from Type II S organisms. These were in each case transformed to Type III S pneumococci. It was found that the substance was active in a final dilution of 1 in 600,000,000, 0.2 milliliters being used, which contained 0.003 microgram of the purified substance.

It was thought that possibly the substance which was actually responsible for the activity of the transforming substance was still unknown, and that it might be adsorbed, or in some other manner be attached to the desoxyribonucleic acid molecule. The investigators therefore looked about for a suitable specific biological tool with which they might destroy or inactivate the desoxyribonucleic acid fraction of the transforming mixture, if indeed there be such a mixture, thereby leaving the true transforming substance intact. It had long been known that enzymes were among the most specific of biochemical reagents at our

disposal at present. These orggnic catalytic agents are even more specific than antigen-antibody reactions. It had been noted, for example, that Type VIII S pneumococci showed cross-reactions in the presence of Type III S anti-pneumococcal serum. During the study of this phenomenon, Dubos had isolated from a bacillus found in the soil, the S III bacillus, an enzyme which was capable of destroying the capsular polysaccharide of pneumococcus Type III, and which had no effect upon the capsular polysaccharide of Type VIII. Similarly, an enzyme was found which attacked only the Type VIII specific polysaccharide, leaving the Type III specific substance intact.(Sickle and Shaw)

In 1940 Kunitz described the method of isolation and the properties of an enzyme, ribonuclease, which he had found in beef pancreas. This enzyme was found to be specific in its action upon ribonucleic acid (yeast type), having no effect upon desoxyribonucleic acid (thymus type). In 1944, McCarty, also working with beef pancreas, isolated an enzyme which was found to be specific in its action upon desoxyribonucleic acid, and was ineffective against nucleic acids of the yeast type. This enzyme was called desoxyribonuclease. It was this substance that was used to determine whether or not the desoxyribnucleic acid fraction isolated from Type III S pneumococcal cultures was actually the inducing substance of the transforming material, or whether it acted merely as a carrier for that substance. From the previous experiments, particularly the electrophoretic and ultracentrifugal methods, it was established that the active substance was a fairly homogeneous material. Accordingly, ex-

periments were set up to determine whether or not the fraction isolated from the pneumococcus containing the activating principle was indeed inactivated after treatment with the new enzyme desoxyribonuclease. It was found that as little as 0.01 microgram of the enzyme completely inactivated the transforming substance. This evidence led quite conclusively to the fact that the actual transforming substance involved in the transformation of pneumococcal types was a nucleic acid of the desoxyribose type, and, moreover, that the substance isolated from the pneumococcus culture extracts was a highly purified compound.

The inactivation of desoxyribonucleic acid may be brought about by a number of chemical agents and biochemical processes. The principal method studied was that of autoxidation.(Avery, McLeod, and McCarty, and others) It was found that ascorbic acid was effective in inactivating the desoxyribonucleic acid of the pneumococcal transformation system. The addition of substances containing sulfhydryl groups was observed to inhibit the inactivating property of the ascorbic acid. This may have been due to the oxidation of sulfhydryl groups, -SH, to the disulfide linkage, -S-S-. In other words a transfer of the activity of the ascorbic acid oxidizing mechanism was effected from one substrate to another, perhaps to one more readily capable of oxidation. It was further observed that the enzyme catalase also inhibited the action of ascorbic acid. When hydrogen peroxide was added to a preparation of desoxyribonucleic acid, ascorbic acid, and catalase, the desoxyribonucleic acid was inactivated. Other peroxides were also effective in inactivating the transforming substance.

but it was seen that none of these, including hydrogen peroxide, were as effective as inhibitors as was the ascorbic acid. The investigators concluded that the mechanism of inhibition was one of autoxidation. Cupric ion, which is known to increase the rate of autoxidation of ascorbic acid, when added to the inhibiting system, notably increased the effectiveness of the ascorbic acid as an inactivator of desoxyribonucleic acid, even when the cupric ion was present to the extent of 0.00001 mole per liter.

It is extremely interesting to note here that the pneumococcus elaborates not only a serologically specific nucleic acid of the desoxyribose type, but also catalase, hydrogen peroxide, and desoxyribonuclease. These substances, the possible interaction of which may readily be seen, serve to illustrate, in a minute way, the complexities of the physiological and biochemical processes of bacterial cells. The four substances may be separated into two systems, each consisting of an enzyme and its substrate, the one, hydrogen peroxide and catalase, and the other desoxyribonucleic acid and desoxyribonuclease. These two systems are linked together by the fact that the hydrogen peroxide acts as an inhibitor of the desoxyribonucleic acid.

These four substances are of interest in connection with their roles in the dissociation of S to R forms and their reversion. It was noted (Avery, McLeod, and McCarty) that serum as a component of the culture medium used in the transforming system is essential. It would therefore seem reasonable to conclude that there are in normal serum one or more substances which have an important role in the process of transformation. When, for

example, Type III S pneumococci are grown in normal serum-containing culture media for a long time, through many transplants, they are seen to lose the property of elaborating the capsular polysaccharide. There may therefore be assumed to be a lack of desoxyribonucleic acid formation. This is found to be so. The mechanism may be either the inactivation of the original amount present by desoxyribonuclease or by the hydrogen peroxide. However it is known that regardless of its phase of dissociation the pneumococcus elaborates the enzyme catalase. This would undoubtedly destroy the hydrogen peroxide formed by the organism, for, because of its specificity and biochemical activity it has no other substrate upon which it may act. This apparently leaves the desoxyribonuclease-desoxyribonucleic acid enzyme-substrate system. It has been noted (Diehl and others) that many of the enzymes which bacterial species elaborate are adaptive, i.e., they are produced by the organism only when a specific substrate is present; the substrate may be only a simple compound containing a particular linkage, such as $-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{N}}-$, or it may be a complex organic compound such as a protein or a carbohydrate. A search of the literature has failed to reveal any data as to whether or not desoxyribonuclease is present in cultures of non-type-specific R pneumococci, such cultures being known to contain a desoxyribonucleic acid fraction which is serologically inactive, and possesses no transforming properties. It would be of interest to know if such a situation exists.

It was found (Avery, McLeod, and McCarty) that when Type II R pneumococci had been transformed to Type III S pneumococci

after treatment with the purified activating substance extracted from cultures of Type III S pneumococci, the transforming substance is transmitted in series and can subsequently be recovered in amounts far in excess of that originally used as inoculum. This phenomenon is strikingly analogous to that exhibited by viruses and genes. Many viruses are known to be pure protein molecules, and genes also are thought to be complex chemical entities closely related to the nucleoproteins. The work of Caspersson and others has pointed to the fact that chromosomes and genes are apparently mixtures of nucleic acids of the ribose and desoxyribose types. There appears to be a balance between these two types of substances set up by the individual cell according to its state of biological and physiological activity.

It is difficult to say at this point exactly what is the relationship existing between the phenomena of bacterial variations and cell mutations on the one hand, and the role of the nucleoproteins in the phenomena of pneumococcal transformation and the self-duplication of viruses and genes in successive generations on the other. It is known, for example, that certain unknown substances apparently present in serum are necessary in the pneumococcal transforming system. The data so far obtained are interpreted as indicating that the serum factors act by altering the su rface of the bacterial cell so that the specific desoxyribonucleic acid is taken up or absorbed. This problem is still in the process of being worked out. The mechanism of the action of the transforming substance upon the metabolic processes of the pneumococcus is yet to be discovered. It is known that

nucleoproteins of both the ribose and desoxyribose types play important roles in the transmission of hereditary characteristics by way of the chromosomes and genes present in the sex cells of plants and animals, as shown in numerous experiments performed with the giant fruit fly Drosophila. But whereas the mutations in the case of Drosophila have been effected by the application of foreign chemical compounds, particularly of the hydrocarbon group, as phenanthrene, pneumococcal transformation has been brought about by the application of a substance known to be normally present, not only in the cells of the pneumococcal species, but also in all other bacterial species and in all plant and animal cells.

The problem of the presence or absence of a nucleus in bacterial species is still an unanswered one, but there is hope that with the aid of the electron microscope, together with spectrometric and physico-chemical techniques, we are at least coming closer to an experimental solution to this problem. It has been observed that a wide variety of bacterial species give a positive Feulgen nucleal reaction, but this technique is still under much controversy. Careful utilization of the nucleal reaction has convinced many authors that bacteria contain well-defined Feulgen positive structures which divide before cellular division, which react like chromatin with the basic dyes, and which are comparable to the chromosomes of plant and animal cells. These structures have been termed nucleoids, chromatinic bodies, or chromosomes. They occur in spores as well as in vegetative forms, and are believed to contain the hereditary mechanism of

the bacterial cell. As pointed out previously in the introduction to this paper, the determination of the presence or absence of a nucleus, and further of a process of nuclear division, or possibly of chromosomic division would be a great advance in the study of the processes involved in karyokinesis and in the transmission in series of chromosomes and genes from one generation to the next. For we pointed out before that in the case of a bacterial species, e.g., Diplococcus pneumoniae, we have an entire evolutionary process or cycle before us in the short space of two or three days, a single cell producing 40 to 50 generations, or about 5×10^{15} organisms, within 72 hours. It would require about 1000 years to produce this number of organisms in the species Homo sapiens, provided they all survived.

Since the different components and properties of the bacterial cell can vary independently of one another, it is possible to obtain a large number of variant forms which, as we have repeatedly emphasized, can be used as reagents in the analysis of bacteriological phenomena. The comparative study of the different variants of one given culture has so far been largely limited to their morphological structures, but there is little doubt that it could apply also to their biochemical processes. It would be interesting to know, for instance, whether the absence of the type-specific polysaccharides or proteins in certain variants of the salmonellae, pneumococci streptococci, etc., is due to the fact that these substances are not produced, or to the fact that they are metabolized further, and thus cannot accumulate.

This is indeed the most striking phenomenon revealed by the study of bacterial variability. The cell can live successfully and continue its existence and multiply as an independent living object after having lost a great variety of structures and functions which had appeared to constitute important components and attributes of the "normal" parent form. These structures and functions can be lost and regained independently of one another, without altering the essential nature of the germ, or the potentialities of the cell. It is even possible to substitute experimentally one character for another, to cause, for instance, a strain of pneumococcus to produce, and to transfer to its progeny the ability to produce, a polysaccharide different from the one it has been known to synthesize heretofore. Not only does the cell appear as an integrated complex of independent characters, but it is possible to substitute for one of these characters another one homologous, but different, without interfering essentially with cellular organization.

All living objects, whatever their nature or dimensions, obey the same natural laws; it is not doubted that the study of bacteria, like that of other cells, will progress with the understanding of the physicochemical phenomena which are the manifestations of their living processes. But each science has, in addition to that fund common to all departments of knowledge, its particular genius determined by the peculiarities of the material which it studies. The extraordinary plasticity of bacteria, the ease with which they adapt themselves to the environment, has not only determined their importance in the eco-

nomy of nature; it also makes them ideal objects for the study of that organization and integration of independent characters which define and characterize life.

ABSTRACT

The study of bacteriological phenomena is the study of many millions of organisms acting simultaneously. It is known that mutations occur naturally in 1 out of 10^5 or 10^6 cell divisions, and though this natural frequency cannot be increased, the survival rate of the mutant form can be increased by various artificial cultural methods. By studying these mutations and the effects that may be produced upon their biochemical, physiological, serological, structural, antigenic and morphological manifestations and processes, we may eventually approach the solution of some of the problems of medical bacteriology in the fields of epidemiology, histology, and cytochemical studies of higher plant and animal cells.

Bacteria show marked pleomorphism even within a single bacterial species. These variations are due to a number of different factors; in the case of the bacillary forms there appears to be an internal axially disposed force which, depending upon the surface tension of the surrounding medium, tends to counteract the rounding effect of that tension upon the cells, thus determining the shape of the cells. The period of growth has a determinative effect, the cells usually being larger or longer in the logarithmic phase of growth. This last factor is also interdependent upon the factor of bacterial nutrition, and upon certain substances or factors that may be present in culture media. For example, lanceolated diplococci of Type III S pneumococcus grown for a long time in Type III S antiserum will exhibit long rods

and filamentous forms. Practically all bacteria exhibit slight morphological variations during their normal growth curve, though some may show such marked variations as in their normal grouping, the loss of certain structures, such as capsules and flagella, and variations in the internal structure of the cell.

Colonial variation within a species indicates the occurrence of mutant forms. Colonial morphology is largely dependent upon the structure of the organisms comprising the colony, and to a lesser dgree upon their biochemical and physiological properties. Colonial organization can be related to the method of growth of the organism, by correlating it with three types of post-fissional movement, snapping, whipping, and sliding. It has been observed that all bacterial species exhibit several types of morphologically distinct colonial forms. These are stabilized through successive generations towards one phase or another. Colonial morphology may be associated with other bacterial properties, such as virulence, encapsulation, enflagellation, and motility. In the case of certain species, colonial morphology may be associated with variations in serological and biochemical manifestations. Three distinct colonial types may be described. The M, or mucoid consists of virulent, encapsulated organisms. The S, or smooth consists of non-capsulated, possibly flagellated organisms which, depending upon the species are virulent or avirulent. The R, or rough consists of non-capsulated, non-flagellated, avirulent organisms. These rules are not hard and fast, for we have the S and R forms of Klebsiella pneumoniae that are encapsulated, and the smooth non-motile and rough mot-

tile variants of the Colon-Salmonella organisms.

The variations of the pneumococcus which were discussed were those of colonial formation, immunochemical specificity, and the transformation of pneumococcal types. A brief historical outline of the studies of the pneumococcus has been given, including the work of Collins, Hiss, and Park and Williams, the serological classification of the organisms by Neufeld and Levinthal and the transformation of types in vivo and in vitro, which were effected by Griffith, Alloway, Dawson and Sia, and Avery, McLeod and McCarty. Three phases of colonial dissociation were found to be associated with the pneumococcus. The M, or mucoid phase consists of encapsulated, virulent organisms showing type specificity. The S, or smooth phase consists of non-capsulated, avirulent organisms, showing species specificity, and retaining the property of reverting to the mucoid phase. The R, or rough phase consists of non-capsulated, avirulent organisms, exhibiting marked pleomorphism, showing long rods and filamentous forms, often negative to the gram stain, and no longer capable of reverting to the smooth or mucoid phases. The rough phase was also shown to be insoluble in bile, and in some cases it was incapable of fermenting inulin.

The work on the transformation of pneumococcal types is based upon the experimental data set forth in 1917 by Dochez and Avery, that each pneumococcal type elaborates a specific soluble substance, i.e., its capsular polysaccharide, which is peculiar only to that type. These substances were isolated in pure form from cultural extracts of pneumococci, and analyzed by a number

of workers who observed that while each of these substances gave positive precipitin tests with their respective antipneumococcal sera, they were of themselves non-antigenic. Seventy-five types of capsular polysaccharides have been differentiated. Certain other variations of the pneumococcus have been observed to be associated with variations in capsular elaboration, namely, resistance to phagocytosis, virulence, type specificity, and colonial morphology. Griffith found in 1923 that R forms of the pneumococcus are identical in all their properties, regardless of the S type from which they were derived, and dependent upon the extent of dissociation. When these R forms were passed through mice, or were grown in ani-R serum, S forms were obtained whose type specificity was that of the S organisms from which the R form was derived, and never of any other type. Alloway reported the same results when R forms were grown in immune anti-R serum. He further found that R forms of one type grown in cell-free extracts of a heterologous S type were transformed to S forms of the type specificity of the cell-free extract. This type specificity was seen to be transmissible in series.

In 1944 Avery, McLeod and McCarty isolated the active transforming substance from cultures of Type III S pneumococci. This substance was analyzed and found to be a nucleic acid of the desoxyribose type. Chemical, physical and biological tests were used to determine the purity of the activating substance. Several substances, notably those which were able to set up autoxidative systems, such as ascorbic acid, were found to inac-

tivate the transforming substance. These inactivating reactions were found to be reversible, except when the desoxyribonucleic acid was inactivated by treatment with desoxyribonuclease, an enzyme isolated from beef pancreas, which irreversibly destroys the nucleic acid. The pneumococcus was observed to elaborate not only the inducing substance, but also several of the inactivating substances. Several factors in serum were found to be essential to the transforming system. The role of enzymes as biological tools in histological and cytochemical studies has been pointed out. The transformation of pneumococcal types and the subsequent transmission of the new hereditary unit has been compared histologically and physico-chemically with the self-duplication of viruses and genes. The problem of a nucleus or of nuclear material in bacteria is discussed briefly in connection with the substances involved in the transformation of pneumococcal types.

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